THE RHODOPSIN SYSTEM OF THE SQUID*

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ABSTRACT

Squid rhodopsin (λ_{max} 493 mμ)—like vertebrate rhodopsins—contains a retinene chromophore linked to a protein, opsin. Light transforms rhodopsin to lumi- and metarhodopsin. However, whereas vertebrate metarhodopsin at physiological temperatures decomposes into retinene and opsin, squid metarhodopsin is stable.

Light also converts squid metarhodopsin to rhodopsin. Rhodopsin is therefore regenerated from metarhodopsin *in the light*. Irradiation of rhodopsin or metarhodopsin produces a steady state by promoting the reactions,

$$\text{Rhodopsin} \xleftarrow{\text{light}} \text{metarhodopsin}$$

Squid rhodopsin contains neo-*b* (11-*cis*) retinene; metarhodopsin all-*trans* retinene. The interconversion of rhodopsin and metarhodopsin involves only the stereoisomerization of their chromophores.

Squid metarhodopsin is a pH indicator, red (λ_{max} 500 mμ) near neutrality, yellow (λ_{max} 380 mμ) in alkaline solution. The two forms—*acid* and *alkaline metarhodopsin*—are interconverted according to the equation,

$$\text{Alkaline metarhodopsin} + H^+ \rightleftharpoons \text{acid metarhodopsin},$$

with pK 7.7. In both forms, retinene is attached to opsin at the same site as in rhodopsin. However, metarhodopsin decomposes more readily than rhodopsin into retinene and opsin.

The opsins apparently fit the shape of the neo-*b* chromophore. When light isomerizes the chromophore to the all-*trans* configuration, squid opsin accepts the all-*trans* chromophore, while vertebrate opsins do not and hence release all-*trans* retinene.

Light triggers vision by affecting *directly* the shape of the retinene chromophore. This changes its relationship with opsin, so initiating a train of chemical reactions.

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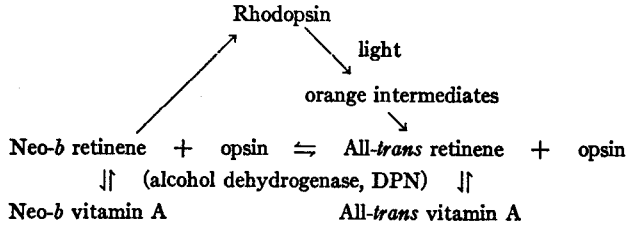
Vertebrate rhodopsin is involved in a cycle of the form (Hubbard and Wald, 1952–53):



In the light rhodopsin bleaches through orange intermediates—lumi- and metarhodopsin—to a mixture of the yellow carotenoid, all-*trans* retinene, and the colorless protein, opsin. Alcohol dehydrogenase and reduced DPN then reduce all-*trans* retinene to all-*trans* vitamin A. To regenerate rhodopsin, a hindered *cis* isomer of retinene or vitamin A is required—neo-b (11-*cis*). The stereoisomerization of retinene and vitamin A therefore is an integral part of the rhodopsin cycle.

The rhodopsin system of the squid differs from the vertebrate system in several respects: (1) Squid rhodopsin in the retina or in (neutral) solution *does not* bleach in the light (*cf.* Krukenberg, 1882; Bliss, 1942–43, 1948), due to the fact that light does not convert squid rhodopsin to retinene + opsin.[1] Instead, the majority of the retinene remains tightly bound to opsin, and can be extracted only with such denaturing organic solvents as acetone or chloroform (*cf.* Wald, 1941, 1942). (2) Wald (1941, 1942) showed that the vitamin A content of the squid retina is low and independent of the state of light or dark adaptation. He therefore concluded that vitamin A does not participate in the rhodopsin system of the squid.

Bliss (1942–43, 1948) claimed that squid rhodopsin was not photosensitive, since it did not bleach in the light.[1] Its photosensitivity, however, was established by St. George and Wald (1949), who showed that in the cold (−45°C.) squid rhodopsin, like vertebrate rhodopsin, is converted by light into lumirhodopsin, which is transformed to metarhodopsin at about −15°C. However, squid metarhodopsin is stable up to about 20°C., whereas vertebrate metarhodopsin above about 0°C. rapidly yields a mixture of retinene + opsin, and regenerated rhodopsin and isorhodopsin (Wald, Durell, and St. George, 1950).

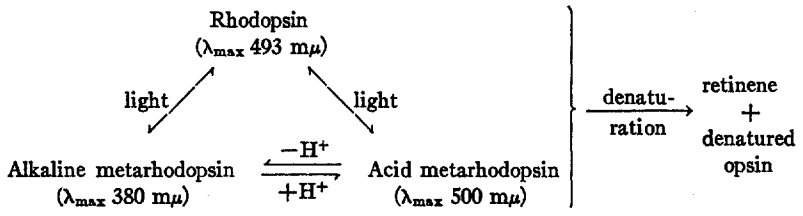
The reactions of squid rhodopsin and metarhodopsin have now been reex-

[1] A distinction must be drawn between the terms *photosensitivity* and *bleaching*. *Bleaching* implies a change in hue or saturation, while *photosensitivity* merely implies a responsiveness to light which need not be accompanied by an observable change in color. In the present paper, bleaching always refers to a change in hue, such as that observed when vertebrate rhodopsin is converted to retinene + opsin.

amined, and may be summarized as follows: (1) In the light squid rhodopsin (λ_{max} 493 mμ) is converted to metarhodopsin, which at temperatures above 15–20°C. slowly dissociates to retinene + opsin. (2) Squid metarhodopsin exists in two forms depending on pH. It is red (λ_{max} 500 mμ) in neutral and mildly acid solution, and yellow (λ_{max} 380 mμ) in alkaline solution. We have called the two forms *acid* and *alkaline metarhodopsin.* They are freely interconvertible in the dark by shifting the pH; the interconversion is half-complete at pH 7.7 (p*K*). (3) Irradiation of squid rhodopsin in neutral or slightly acid solution yields acid metarhodopsin—hence no bleaching.[1] Irradiation in alkaline solution bleaches, since it yields alkaline metarhodopsin. (4) The conversion of squid rhodopsin to either form of metarhodopsin does not release retinene, but involves the isomerization of the chromophore from the neo-*b* to the all-*trans* configuration. (5) Alkaline metarhodopsin, though it resembles retinene spectroscopically, can be readily distinguished as a separate chemical entity. It is composed of retinene firmly bound to opsin at the same site to which it is attached in acid metarhodopsin and rhodopsin. (6) Under the conditions explored so far, squid rhodopsin cannot be regenerated *in the dark* from metarhodopsin or from retinene + opsin. However light, in addition to converting squid rhodopsin to metarhodopsin, brings about also the reverse reaction, the regeneration of rhodopsin from metarhodopsin. (7) Continuous irradiation of squid rhodopsin *or* metarhodopsin therefore produces a steady state, independent of the intensity of the light:

$$\text{Rhodopsin} \underset{}{\overset{\text{light}}{\rightleftharpoons}} \text{metarhodopsin}$$

At pH 6 this contains roughly equal amounts of rhodopsin and acid metarhodopsin (actual proportions 0.9:1). (8) Analyses of retinas from light- and dark-adapted squid indicate that *in vivo* the retina possesses also mechanisms for converting metarhodopsin to rhodopsin *in the dark.* We have not yet succeeded in demonstrating these *in vitro.* (9) The squid rhodopsin system may be summarized as follows:—



These reactions will now be described in more detail.

Experimental Methods

Preparation of Squid Rhodopsin.—Squids were decapitated and the heads bisected, permitting the eyes to be extruded from their orbits. The optic ganglion was trimmed

away and the eye hemisected, exposing the retina which was lifted out with a spatula. Retinas were collected only from freshly killed squid. After dissection, they were sometimes frozen and stored at −15°C. No differences were observed between extracts from fresh or frozen retinas. The dissection was carried out in red or dim white light, the preparation of rhodopsin always in red light.

The retinas were ground with M/15 phosphate buffer (pH 6.1), and centrifuged in a refrigerated Spinco preparative centrifuge for 5 minutes (No. 40 rotor at 30,000 R.P.M.). The supernatant, containing a red water-soluble pigment which bears no relation to rhodopsin, was discarded. The tissue was disintegrated by stirring in a plastic Spinco centrifuge tube (12 ml. capacity), adding sucrose solution (36 per cent *w/v*, in M/15 phosphate buffer, pH 6.1) in small portions, and grinding after each addition. The final suspension, in about 4 ml. sucrose solution per retina, was layered under phosphate buffer and centrifuged 10 minutes at 30,000 R.P.M. The rods float to the sucrose-buffer interface, the retinal debris sediments. The rods were drawn off, the residue reground with sucrose, and the flotation repeated. The rods from the two flotations were pooled, sedimented from buffer, and washed twice with 0.1 M Na_2HPO_4 and once with distilled water (10 ml. each), centrifuging each time for 5 minutes at 30,000 R.P.M. Rhodopsin was then extracted by leaching the rods about 1 hour with 2 per cent digitonin in phosphate buffer (M/15, pH 6.1), using about 0.5 to 1 ml. solvent per retina. The suspension was centrifuged 10 minutes at 20,000 R.P.M. and the clear supernatant, containing the rhodopsin, drawn off. The rods were reextracted until they yielded no further rhodopsin. All solutions were made up in glass-distilled water, and the entire procedure carried out on ice.

This method routinely yielded solutions of high optical purity (K_{min}/K_{max} = 0.30 ± 0.05). However, squid rhodopsin is less stable than cattle rhodopsin: kept dark in the refrigerator, it slowly bleaches irreversibly to retinene and opsin. It was therefore usually prepared in small lots and used within about a week.

Interconversion of Rhodopsin and Metarhodopsin.—Rhodopsin and metarhodopsin were irradiated with a 160 watt microscope lamp shielded at all times by a 2 cm. water cell and a heat filter (Corning *3962* or *3966*). In addition, an orange filter (Corning *3482*) was used to bleach rhodopsin to alkaline metarhodopsin; Corning *3482* or *3389* (pale yellow) filters for the interconversion of rhodopsin and acid metarhodopsin; and a Jena *UG 2* filter, transmitting only the near ultraviolet, to irradiate alkaline metarhodopsin.

The solutions were immersed in ice water at all times, except when spectra were measured. For spectrophotometry, the cell compartment of a Beckman spectrophotometer was kept at 5°C. by circulating ice water through Beckman thermospacers. The cell carriage and absorption cells were precooled in the cell compartment and kept there during the entire experiment. The solutions (0.4 to 0.5 ml.) were introduced and withdrawn with chilled Carlsberg constriction pipettes (Glick, 1949, pp. 172–173).

pH was measured with a Beckman pH meter using a 0.6 ml. cell. To avoid dilution of the samples, the pH was adjusted with solid Na_2CO_3 or KH_2PO_4, or with CO_2. It has been reported that high concentrations of salt stabilize the orange intermediates when bleaching vertebrate rhodopsin (Bridges, 1956). The salt concentrations employed in our experiments do not have this effect.

Analysis of Mixtures of Rhodopsin and Acid and Alkaline Metarhodopsin.—If rhodopsin in neutral or slightly alkaline solution is irradiated with orange light, mixtures are formed which contain these three components (see text below, Table III). They can be analyzed by the following procedure: the absorption spectrum of the rhodopsin solution is measured initially (1) and after irradiation (2). Spectrum 2 is that of the mixture to be analyzed. All metarhodopsin in the mixture is now converted to the alkaline form by raising the pH to 9.7, and the spectrum is remeasured (3). The mixture—now containing only rhodopsin and alkaline metarhodopsin—is reirradiated with orange light to convert the rhodopsin to alkaline metarhodopsin, and the spectrum is measured (4). The solution now contains only alkaline metarhodopsin. The difference in extinction at 500 mμ between spectra 2 and 3 is the extinction of acid metarhodopsin in the mixture; the difference at 493 mμ between spectra 3 and 4, the extinction of rhodopsin. Since alkaline metarhodopsin has essentially no absorption above 450 mμ, these differences are the true extinctions of rhodopsin and acid metarhodopsin. They are converted to concentrations using the molar extinction coefficients[2] of rhodopsin and acid metarhodopsin (Table I). These have been determined as described below. The contribution of alkaline metarhodopsin to spectrum 2 is best estimated by subtracting the concentrations of rhodopsin and acid metarhodopsin in the mixture from the initial rhodopsin concentration (the difference in extinctions at 493 mμ between spectra 1 and 4). This is preferable to computing the concentration of alkaline metarhodopsin directly from the extinction at 380 mμ, which may be contaminated with colored impurities. The concentrations of rhodopsin and acid metarhodopsin are calculated from *differences* in extinction, hence are independent of the optical purity of the solution.

TABLE I

Absorption Properties of Rhodopsin and Acid and Alkaline Metarhodopsin in Aqueous Digitonin

The wavelength of maximum extinction (λ_{max}) and the molar extinction coefficient (ϵ_{max}) at this wavelength.[2] ϵ_{max} was determined as described under Methods.

Compound	Absorption properties	
	λ_{max}	ϵ_{max}
	mμ	*cm.²/mole*
Rhodopsin	493	40,600
Acid metarhodopsin	500	59,700
Alkaline metarhodopsin	380	60,900

Thermal Bleaching of Rhodopsin and Acid Metarhodopsin.—The temperature of the cell compartment of the Beckman spectrophotometer was regulated by circulating water from a constant temperature reservoir through the thermospacers. The tempera-

[2] The molar extinction coefficient (ϵ) is defined as the extinction of a 1 M solution in a layer of 1 cm. depth, and expressed in cm.² per mole. The molar extinction coefficient at the wavelength of maximum absorption (λ_{max}) will be designated ϵ_{max}.

ture of the reservoir was controlled to within 0.1°C. with a bimetallic thermoregulator (American Instrument Company). The temperatures of the reservoir and the cell compartment were identical near room temperature. At the higher temperatures, the temperatures of bath and cell compartment were related as shown in Fig. 1. The temperature of the cell compartment was measured by inserting a thermometer through cotton wool before and after each experiment (Fig. 1, solid circles), as well as during a final calibration (Fig. 1, open circles). The two sets of data agree to within

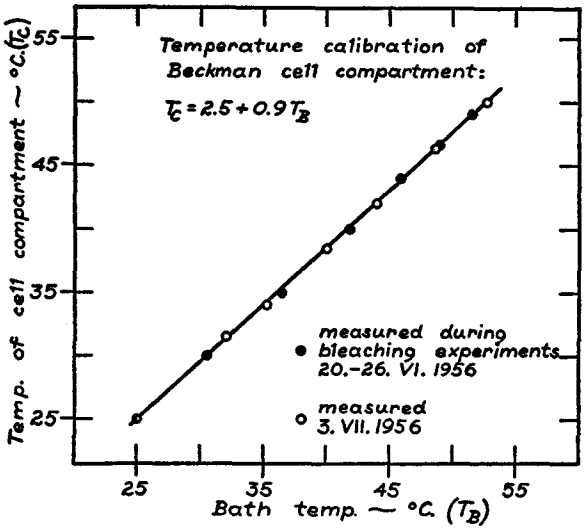


FIG. 1. Temperature calibration of the Beckman cell compartment. The temperature of the cell compartment was regulated by circulating water from a constant temperature bath through thermospacers. The temperatures of the bath and cell compartment are related as shown in the figure. They were measured before and after each experiment (solid circles) as well as during a final calibration (open circles). The two sets of data lie on the same straight line, which yields the relationship, $T_c = 2.5 + 0.9\,T_B$, in which T_c is the temperature of the cell compartment and T_B, the temperature of the bath.

0.5°C., which represents the accuracy of this method. The temperature was monitored during each experiment with a thermometer inserted into the block to the monochromator side of the thermospacer. At high temperatures, the block is 1–2° cooler than the cell compartment, but its temperature can be used to check the constancy of the temperature during each experiment.

For measurements of the rate of thermal decomposition, the cell holder and absorption cells containing all ingredients but rhodopsin or acid metarhodopsin were equilibrated in the prewarmed cell compartment for about an hour. The samples were then introduced and the extinction at λ_{max} followed. The rates of destruction increased during the first 1 or 2 minutes, while the samples came to temperature equilibrium; from then on they were constant until all pigment had been bleached (*cf.* Fig. 8).

Determination of Vitamin A and Free and Protein-Bound Retinene.—The retinas were ground with anhydrous sodium sulfate and extracted by stirring with several successive portions of petroleum ether, centrifuging each time. These extracts contained all the vitamin A and free retinene. The protein-bound retinene was extracted by stirring the retinal powder with chloroform or with acetone containing about 1 per cent water. The extracts were transferred to chloroform, and the amount of vitamin A and retinene determined by the antimony chloride reaction.

Molar Extinction Coefficients of Rhodopsin, and the Two Forms of Metarhodopsin, and the Rhodopsin Content of the Squid Retina.—The molar extinction coefficients[2] of rhodopsin, and acid and alkaline metarhodopsin at their respective λ_{max} were determined as follows: as will be seen below, the chromophores of rhodopsin and metarhodopsin can be converted to all-*trans* retinene oxime in a variety of ways. The molar extinction coefficient (ϵ_{max}) of all-*trans* retinene oxime is 51,600 (Wald and Brown, 1953–54). Several experiments yielded an average value of 0.79 for the ratio of extinctions, squid rhodopsin:all-*trans* retinene oxime. The molar extinction coefficient (ϵ_{max}) of squid rhodopsin therefore is 40,600, the same as for cattle rhodopsin (Wald and Brown, 1953–54). The extinctions of acid and alkaline metarhodopsin are respectively 1.47 and 1.50 times higher than that of rhodopsin (*cf.* Fig. 2). The two forms of metarhodopsin therefore have molar extinctions (ϵ_{max}) of 59,700 and 60,900 (*cf.* Table I).

One squid retina yields on the average 1 ml. of a rhodopsin solution with an extinction at λ_{max} of 1.0. Since the ϵ_{max} of squid rhodopsin is 40,600, this is equivalent to a rhodopsin content of 0.025 micromole per retina. The cattle retina, which has about four times the area of the squid retina, contains about 0.02 micromole rhodopsin. The squid retina is therefore comparatively rich in rhodopsin.

RESULTS

1. Interconversion of Squid Rhodopsin and Acid and Alkaline Metarhodopsin

Effect of pH.—Squid rhodopsin (λ_{max} 493 mμ), upon irradiation with orange light at pH 9.5–10, yields alkaline metarhodopsin (λ_{max} 380 mμ), and hence bleaches.[1] If the pH is now lowered to 5.5 in the dark, alkaline metarhodopsin is converted quantitatively to acid metarhodopsin (λ_{max} 500 mμ). This is the only way to obtain pure acid metarhodopsin from rhodopsin, for reasons discussed below. The reaction sequence is illustrated in Fig. 2; the absorption properties of the three compounds are summarized in Table I.

Rhodopsin may be bleached in alkaline solution as before, and the acidification of metarhodopsin carried out stepwise, in the form of a titration. In this case one obtains such intermediate mixtures of acid and alkaline metarhodopsin as shown in Fig. 3. As the pH is lowered, the absorption due to alkaline metarhodopsin (λ_{max} 380 mμ) decreases while the absorption due to acid metarhodopsin (λ_{max} 500 mμ) rises proportionately. The curves cross at a single

wavelength, the isosbestic point (420 mμ), indicating that the reaction involves only the two substances, alkaline and acid metarhodopsin. As shown in Fig. 4, the data can be fitted by the titration curve of a single ionizable group with pK 7.7. The reaction is therefore described by the equation,

$$\text{Alkaline metarhodopsin} + H^+ \rightleftharpoons \text{acid metarhodopsin.}$$

Low pH favors the formation of acid metarhodopsin; high pH, its dissociation into alkaline metarhodopsin + H^+.

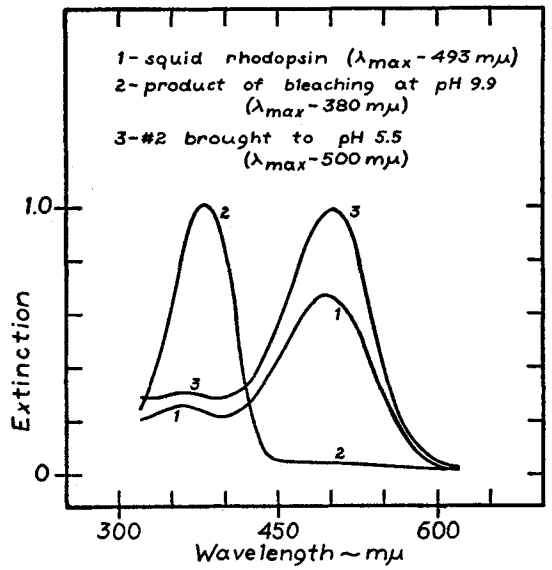


FIG. 2. Absorption spectra of squid rhodopsin (1) and of alkaline (2) and acid (3) metarhodopsin.

As with most ionizations, this equilibrium is temperature-dependent. A decrease in temperature decreases the tendency to ionize and therefore shifts the equilibrium toward acid metarhodopsin. Data from four experiments, summarized in Table II, yield a heat of ionization (ΔH) of about +8000 cal. per mole. This is of the same order of magnitude as the heats of ionization of imidazolium and amino acid ammonium ions (about 7000 and 11,000 cal. per mole, respectively; Cohn and Edsall, 1943, pp. 80, 82, 89).

Alkaline metarhodopsin has in the past been confused with retinene because of spectroscopic similarities (*cf.* St. George *et al.*, 1952; Hubbard and St. George, 1956). The two compounds differ in a number of respects: (1) In alkaline solution, retinene ($C_{19}H_{27} \cdot \underset{H}{C} = O$) in the presence of opsin exists as a Schiff base, $C_{19}H_{27} \cdot \underset{H}{C}{=}N \cdot$opsin, with λ_{max} 365 mμ and ϵ_{max} about 47,000.[2] These spectro-

scopic properties are characteristic of the Schiff bases of retinene (Ball *et al.*, 1949; Pitt *et al.*, 1955). Alkaline metarhodopsin, as we have seen, has λ_{max} 380 mμ and ϵ_{max} 60,900. (2) Neutralization of the Schiff base releases retinene (λ_{max} 385 mμ, ϵ_{max} 35,000), while neutralization of alkaline metarhodopsin yields acid metarhodopsin (λ_{max} 500 mμ, ϵ_{max} 59,700). (3) Retinene combines

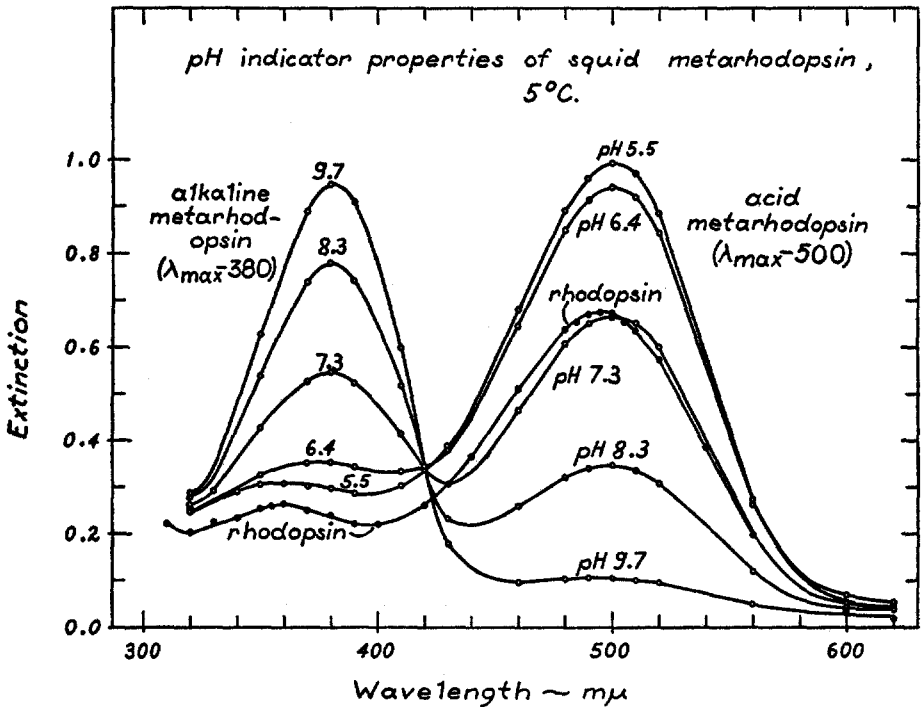


FIG. 3. Absorption spectra of squid rhodopsin; alkaline metarhodopsin, produced by bleaching with orange light at pH 9.7; mixtures of alkaline and acid metarhodopsin, produced by subsequent gradual acidification in the dark (pH 8.3, 7.3, and 6.4); and, finally, pure acid metarhodopsin (pH 5.5).

rapidly with hydroxylamine to form the oxime, $C_{19}H_{27}\cdot C(H)=NOH$, while alkaline metarhodopsin does not react with hydroxylamine. (4) Alcohol dehydrogenase and reduced DPN reduce retinene to vitamin A ($C_{19}H_{27}\cdot CH_2OH$), but do not react with alkaline metarhodopsin.

The aldehyde group of retinene participates in all four reactions just described. It is absent—or unavailable—in squid metarhodopsin, acid or alkaline. Denaturation of metarhodopsin by heat or chemically releases retinene, which then reacts as would be expected.

Effect of Irradiating Metarhodopsin.—Rhodopsin is not regenerated from

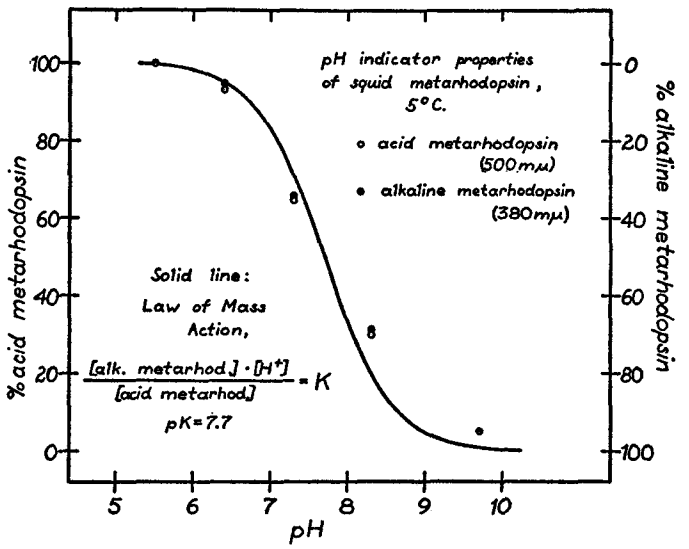


FIG. 4. The data of Fig. 3 replotted to show the change in composition of the mixture of acid and alkaline metarhodopsin as a function of pH. At pH 5.5, the solution contains only acid metarhodopsin, at pH 9.7 almost only alkaline metarhodopsin. The intermediate points are fitted by the titration curve of an ionizable group with pK 7.7.

TABLE II

Heat of Ionization (ΔH) *for the Reaction,*

Acid metarhodopsin $\rightleftharpoons$ *alkaline metarhodopsin* + H^+

ΔH is calculated from the equation,

$$\log \frac{K_1}{K_2} = \frac{\Delta H}{2.303R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right),$$

in which K_1 and K_2 are the observed equilibrium constants at absolute temperatures T_1 and T_2, and R is the gas constant (1.986 cal./mole degrees).

T_1	T_2	$\log \frac{K_1}{K_2}$	ΔH
°K.	°K.		*cal. per mole*
278	293	−0.33	+7900
278	298	−0.39	+7400
279.5	303	−0.36	+7850
279.5	308	−0.47	+8250
Average			+7850

acid or alkaline metarhodopsin *in the dark. Irradiation* of metarhodopsin, however, yields rhodopsin. Fig. 5 shows the result of irradiating alkaline metarhodopsin; Fig. 6 (right), the result of irradiating acid metarhodopsin. In both cases, the rhodopsin is regenerated photochemically; there is no evidence of a

dark reaction. As shown in Fig. 5, the irradiation of alkaline metarhodopsin yields a pigment with λ_{max} 485 mμ, presumably a mixture of regenerated rhodopsin and isorhodopsin (*cf.* Hubbard and Wald, 1952–53). Irradiation of acid metarhodopsin yields rhodopsin uncontaminated with isorhodopsin (Fig. 6, right). Light therefore not only converts rhodopsin to metarhodopsin, but also

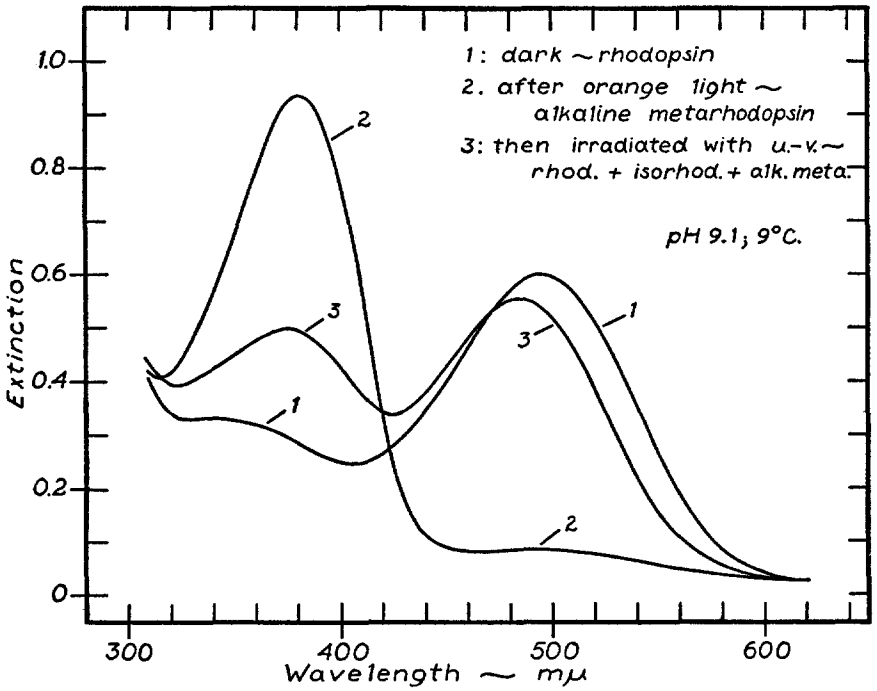


FIG. 5. Rhodopsin (1) upon irradiation with orange light (Corning filters *3482* + *3966*, transmitting $\lambda > 550$ mμ) at pH 9.1 yields essentially pure alkaline metarhodopsin (2). Irradiation of alkaline metarhodopsin with ultraviolet light (Jena *UG 2*, transmitting between 300 and 400 mμ) causes a drop in extinction at 380 mμ and a rise maximal at 485 mμ. This is due to the partial conversion of alkaline metarhodopsin to a mixture of rhodopsin and isorhodopsin. Both changes take place *in the light*, and there is no evidence of dark reactions.

metarhodopsin to rhodopsin. It produces a pseudoequilibrium or steady state of the form:

$$\text{Rhodopsin} \xleftrightarrow{\text{light}} \text{metarhodopsin.}$$

The absorption spectra of rhodopsin and acid metarhodopsin overlap so extensively that light absorbed by one is necessarily absorbed by the other. In neutral or slightly acid solution, the reaction therefore always proceeds in both directions, so that starting with either rhodopsin or acid metarhodopsin,

irradiation produces a mixture of both. In alkaline solution, however, since the absorption spectra of rhodopsin and alkaline metarhodopsin overlap only slightly, it is possible to irradiate each of these substances separately. White light, which contains wavelengths absorbed by both pigments, produces steady state mixtures. Near ultraviolet light, which is absorbed more strongly by alkaline metarhodopsin, results in a large production of rhodopsin (and isorhodopsin; *cf.* Fig. 5), while orange light, absorbed by rhodopsin alone, bleaches

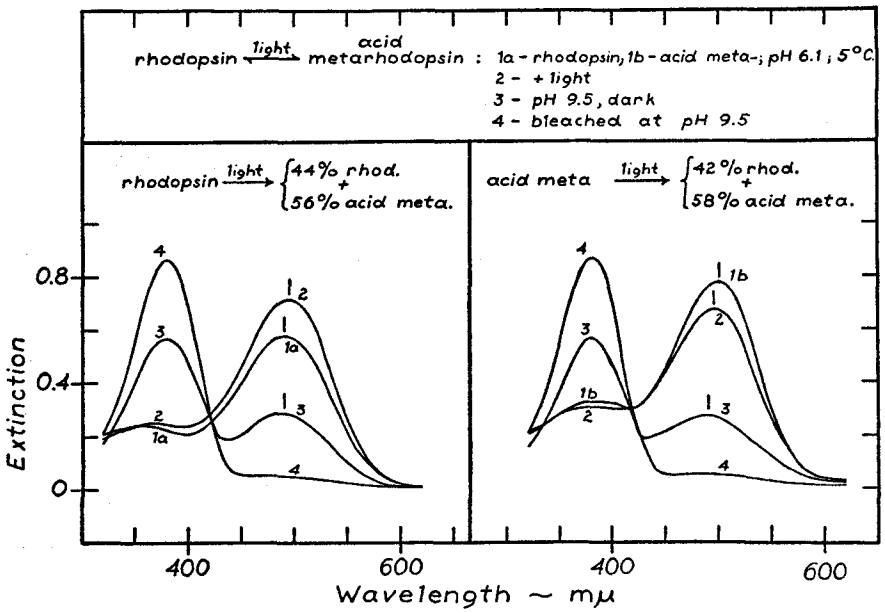


FIG. 6. Irradiation of pure rhodopsin (left) or acid metarhodopsin (right) with orange light yields a mixture containing about 43 parts rhodopsin and 57 parts acid metarhodopsin. The method of analyzing this mixture is described in the text and under Methods.

the latter to alkaline metarhodopsin (*cf.* Figs. 2, 3, and 5). Only in alkaline solution, therefore, and with orange light can rhodopsin be converted completely to metarhodopsin.

The interconversion of rhodopsin and acid metarhodopsin is illustrated in Fig. 6. On the left, rhodopsin (curve 1 *a*) is irradiated with orange light at pH 6.1. There is a *rise* in extinction and a shift of λ_{max} toward *longer* wavelengths (curve 2, left; λ_{max} 496 mμ). On the right, acid metarhodopsin (curve 1 *b*), obtained by bringing alkaline metarhodopsin to pH 6.1, is irradiated in the same way: the extinction *falls* and λ_{max} shifts toward *shorter* wavelengths (curve 2, right; λ_{max} 496 mμ). The pH of both solutions is now raised in the dark,

causing the extinction to fall at 500 mμ and rise at 380 mμ (curves 3, left and right)—acid metarhodopsin is converted to alkaline metarhodopsin. This reveals the presence of rhodopsin *in both cases*. The rhodopsin is finally bleached to alkaline metarhodopsin by irradiation at pH 9.5 (curves 4, left and right). Irradiation of rhodopsin or acid metarhodopsin therefore produces a mixture of both, in the approximate proportion 43:57 (for details of calculation, see Methods).

To go a little further with this relationship: irradiation with *orange* light results in a steady state mixture, which contains the same proportions of rhodopsin and acid metarhodopsin regardless of whatever alkaline metarhodopsin

TABLE III

Composition of Mixtures of Rhodopsin and Acid and Alkaline Metarhodopsin Produced by Irradiation with Orange Light at Various pH's

In parentheses are shown the concentrations calculated on the basis of assumptions described in the text. The agreement between the observed and calculated concentrations is within experimental error.

pH	Rhodopsin	Acid metarhodopsin	Alkaline metarhodopsin	Rhodopsin / Acid metarhodopsin
	per cent	*per cent*	*per cent*	
6.1	44 (47)	56 (52)	Negligible (1)	0.8
6.1*	47 (47)	53 (52)	" (1)	0.9
6.2	42 (47)	58 (52)	" (1)	0.75
7.6	33 (34)	37 (38)	30 (28)	0.9
8.35	21 (19)	19 (22)	60 (59)	1.1
Average				0.9

* This experiment was performed under prepurified nitrogen in order to ascertain whether the reactions were affected by oxygen; all the others in air.

is present. The ratio of rhodopsin to acid metarhodopsin is therefore independent of pH. As shown in Table III, it has an average value of 0.9. However, as already explained, acid metarhodopsin is also in equilibrium with alkaline metarhodopsin, depending upon pH, and independent of light (Fig. 4). The experimentally observed concentrations of all three substances shown in Table III are therefore compared with values in parentheses which have been calculated on the assumption that in orange light (*i.e.* light not absorbed by alkaline metarhodopsin) the steady state is achieved *via* the coupled reactions,

$$\text{rhodopsin} \xleftarrow{\text{light}} \text{acid metarhodopsin} \rightleftharpoons \text{alkaline metarhodopsin} + H^+.$$

The agreement between the observed and calculated values supports this assumption.

Chemical Constitution of Rhodopsin and the Two Forms of Metarhodopsin.—

None of the reactions described so far is inhibited by hydroxylamine (0.2 M) or *p*-chloromercuribenzoate (*p*CMB; 10^{-3} M). The former reagent is known to "trap" free retinene by condensing with its carbonyl group to form retinene oxime; the latter is a relatively specific and powerful sulfhydryl (—SH) reagent. Neither the carbonyl group of retinene, therefore, nor sulfhydryl groups on opsin become available during these transformations. The interconversions of rhodopsin and the two forms of metarhodopsin must proceed without the liberation of retinene, and the attendant exposure of —SH groups on opsin. Yet squid rhodopsin and metarhodopsin differ spectroscopically and in their chemical behavior. What underlies these differences?

Squid rhodopsin and metarhodopsin, though they retain their individuality indefinitely in the dark, are interconverted rapidly by light. The same kind of relationship is known to involve the geometrical isomers of retinene. Each of them is stable in the dark, but is rapidly converted to a steady state mixture with others in the light (*cf.* Hubbard, Gregerman, and Wald, 1952–53; Hubbard, 1956). In the presence of the enzyme, retinene isomerase, this steady state involves specifically the pair of isomers, all-*trans* and neo-*b* retinene (Hubbard, 1955–56). This is the essential relationship involved in the interconversions of squid rhodopsin and metarhodopsin; the transformation by light of either pigment into the other involves a change in the geometric configuration of its chromophore.

What are the stereoisomeric configurations of retinene in squid rhodopsin and metarhodopsin? This could be established most satisfactorily by synthesis from opsin and the appropriate isomer of retinene. Thus the stereoisomeric configurations of the chromophores of vertebrate rhodopsin and its isoartefact, isorhodopsin, were established by showing that they are synthesized by the combination of opsin with neo-*b* (11-*cis*) and iso-*a* (9-*cis*) retinene, respectively (Hubbard and Wald, 1952–53). However, we have not yet succeeded in synthesizing rhodopsin or metarhodopsin from squid opsin and retinene. The reason seems to be that squid opsin is extremely labile, and denatures during or immediately following removal of the chromophore (see below). The configurations of the chromophores of squid rhodopsin and metarhodopsin must therefore be inferred from other evidence.

Rhodopsin has by now been extracted from the rods of a large number of animals, including representatives of all classes of vertebrates. The absorption maxima of these pigments range from about 480 to 503 mμ, due to species differences in the opsin. Yet the chromophore always contains neo-*b* retinene (Wald, Brown, and Brown, 1957).[3] Squid rhodopsin (λ_{max} 493 mμ) has the same molar extinction as cattle rhodopsin. It therefore seems reasonable to assume that the chromophore of squid rhodopsin also contains neo-*b* retinene.

[3] A rhodopsin has been described from *Gecko* lizards with λ_{max} as high as 524 mμ (Crescitelli, 1956–57). The isomeric constitution of its chromophore has not yet been examined.

How about metarhodopsin? Several lines of evidence suggest that the prosthetic group of squid metarhodopsin is derived from all-*trans* retinene. (1) Light isomerizes neo-*b* retinene primarily to the all-*trans* configuration (*cf.* Hubbard, 1956). One would expect it to isomerize neo-*b* retinene acting as a chromophore to the all-*trans* chromophore. (2) The extinction of all-*trans* retinene is much higher than that of neo-*b* retinene (ratio of extinctions 1.7); the extinction of both forms of squid metarhodopsin is similarly higher than that of rhodopsin (ratio of extinctions 1.5). (3) If vertebrate rhodopsin (cattle, frog) is brought into 0.1 M HCl, it is converted to a compound with λ_{max} at 440 mμ—"acid indicator yellow" (*cf.* Lythgoe, 1938; Wald, 1937–38). Morton and Pitt (1955) have shown that this compound (ϵ_{max} 37,700) probably contains *cis* isomers of retinene. When squid rhodopsin is treated in the same way, it yields acid indicator yellow with ϵ_{max} 37,400, in good agreement with Morton and Pitt's value. The presence of *cis* isomers can be demonstrated by exposing the acid indicator yellow to light. There is an immediate rise in extinction, indicating an isomerization from *cis* to the *trans* configuration. If squid *metarhodopsin* is treated with 0.1 M HCl in the dark, an acid indicator yellow is formed, the extinction of which *falls* on exposure to light—indicating isomerization from *trans* to *cis* configurations. This again suggests that metarhodopsin contains all-*trans* retinene.[4]

Squid rhodopsin therefore contains neo-*b* retinene and metarhodopsin all-*trans* retinene.

2. *Relative Stabilities of Rhodopsin and Metarhodopsin*

Only the chromophores of rhodopsin and metarhodopsin have been discussed so far. The properties of opsin, however, also differ in the two compounds. We have examined the behavior of rhodopsin and acid metarhodopsin *in the dark*, under conditions which attack primarily the opsin—*p*CMB, ethanol, formaldehyde, and heat. We have also observed the effect of hydroxylamine, which probably attacks directly the linkage between the chromophore and opsin. In every case, metarhodopsin is more labile than rhodopsin.

pCMB and Hydroxylamine.—The effects of *p*CMB and hydroxylamine are summarized in Figs. 7 and 8. Rhodopsin is stable in the presence of 10^{-3} M *p*CMB or 0.2 M hydroxylamine; acid metarhodopsin is destroyed by both. About half the metarhodopsin is destroyed in 5 hours in the presence of *p*CMB, and 20 per cent in the presence of hydroxylamine. The effects of *p*CMB and

[4] *Note Added in Proof.*—One of us has recently found that thermal denaturation releases the retinene chromophore from opsin without altering its stereochemical configuration. Thus cattle rhodopsin releases predominantly neo-*b* retinene; isorhodopsin, iso-*a* retinene (Hubbard, 1958*a*). By this method we have finally established the configurations of the chromophores of squid rhodopsin and metarhodopsin. Rhodopsin releases predominantly neo-*b* retinene; both forms of metarhodopsin, all-*trans* retinene (*viz.* Hubbard and Kropf, in press).

hydroxylamine are additive, and when both are present two-thirds of the metarhodopsin is destroyed in 5 hours (Fig. 7), and essentially all of it after 26 hours (Fig. 8, right, curve 5).

As shown in Fig. 8 (left, curves 1 to 4), during destruction of acid metarhodopsin by *p*CMB, the fall in extinction at 500 mμ is accompanied by a slow rise in extinction maximal at about 395 mμ. This is due to retinene, which reacts

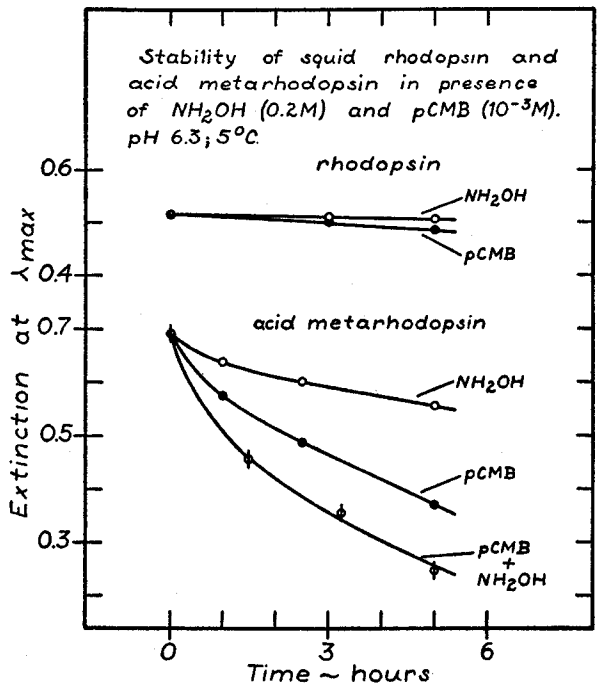


FIG. 7. Effects of hydroxylamine (NH_2OH-0.2 M) and *pCMB* (10^{-3} M) on rhodopsin and acid metarhodopsin. During a 5 hour incubation, rhodopsin is essentially stable, while acid metarhodopsin is gradually destroyed. The effects of the two compounds on acid metarhodopsin are roughly additive.

instantaneously with hydroxylamine forming all-*trans* retinene oxime (left, curve 5). The destruction is stopped but not reversed by excess glutathione, which itself combines with *p*CMB. *p*CMB therefore labilizes the attachment of the chromophore to opsin in metarhodopsin, but not in rhodopsin.

Hydroxylamine (0.2) also destroys acid metarhodopsin though not rhodopsin (Fig. 7), converting the chromophore to all-*trans* retinene oxime (λ_{max} 367 mμ). In the presence of *p*CMB *and* hydroxylamine (Fig. 8, right), the spectroscopic changes are the same as with hydroxylamine alone, since the retinene liberated by *p*CMB reacts with hydroxylamine as rapidly as formed. In Fig. 8

(right, curve 5) the absorption spectrum of all-*trans* retinene oxime in digitonin has been plotted together with the spectrum of the final product of bleaching by *p*CMB plus hydroxylamine. The identity between the two shows that

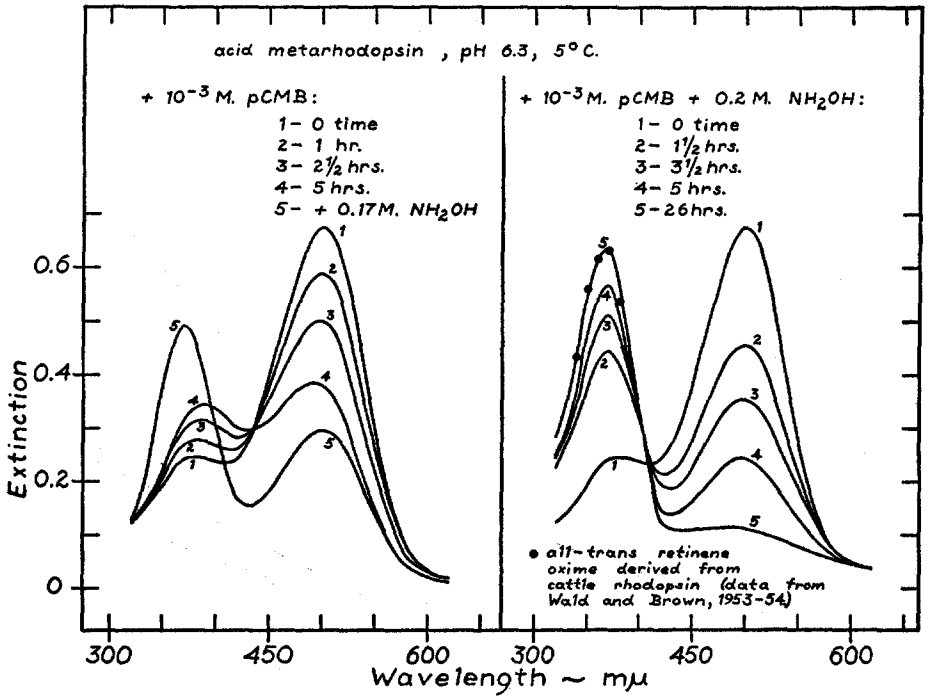


FIG. 8. Left, when acid metarhodopsin is destroyed by *p*CMB, the drop in extinction at 500 mμ is accompanied by a slow rise maximal at about 395 mμ (curves 1 to 4). This is due to the expulsion of retinene from the chromophoric site on opsin. On addition of hydroxylamine, the retinene is instantaneously converted to retinene oxime (curve 5). In time the residual acid metarhodopsin will be completely converted to retinene oxime, as shown on the right. Right, acid metarhodopsin is incubated in the presence of both *p*CMB and hydroxylamine. The spectroscopic picture is the same as with hydroxylamine alone for reasons explained in the text, but the rate of destruction is much faster (*cf.* Fig. 7). After 26 hours, essentially all acid metarhodopsin has been converted to all-*trans* retinene oxime (curve 5). This is shown by superimposing the absorption spectrum of all-*trans* retinene oxime and curve 5. In the region of λ_{max}, the two are identical.

the retinene liberated by *p*CMB and hydroxylamine is in the all-*trans* configuration.

Ethanol and Formaldehyde.—10 per cent ethanol or formalin also bleaches acid metarhodopsin without affecting rhodopsin. During a 3 hour incubation

at pH 6.5 and 5°C., about two-thirds of the metarhodopsin is destroyed with ethanol and one-third with formalin. Both reagents release all-*trans* retinene, which is converted by hydroxylamine to the oxime.

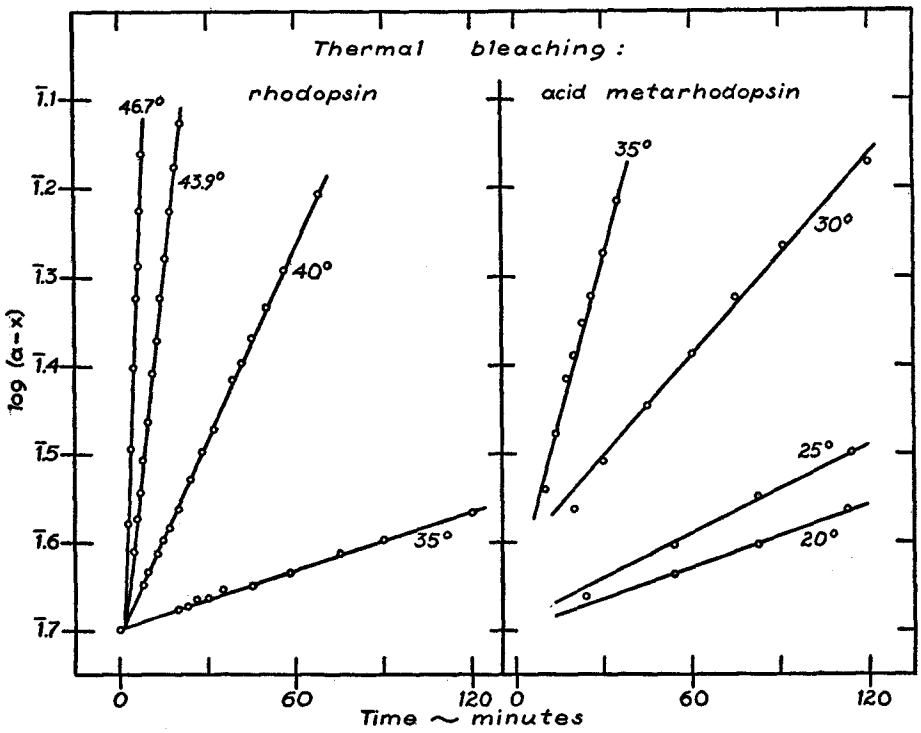


FIG. 9. Thermal bleaching of rhodopsin and acid metarhodopsin. The data are plotted in terms of the equation for a monomolecular reaction in the form, $k = \frac{2.303}{t_1 - t_2} \log \frac{(a - x)_1}{(a - x)_2}$, in which k is the velocity constant, and $(a - x)_1$ and $(a - x)_2$ are the extinctions at λ_{max} of rhodopsin or acid metarhodopsin at times t_1 and t_2. When $\log(a - x)$ is plotted against t, the points lie on straight lines, as would be expected for monomolecular reactions. The velocity constant, k, at each temperature is equal to 2.303 times the slope of the corresponding line. The values of k, determined in this way, are given in Table IV (column II).

The differential sensitivity of acid metarhodopsin and rhodopsin to ethanol and formaldehyde accounts for Bliss's report that these reagents "photosensitize" rhodopsin (Bliss, 1942–43, 1948). Rhodopsin is resistant to them. Exposure to light, however, produces acid metarhodopsin, which is promptly attacked and bleaches.

Thermal Bleaching of Squid Rhodopsin and Acid Metarhodopsin.—Heat irreversibly bleaches rhodopsin and acid metarhodopsin in the dark. However, the threshold for thermal bleaching differs for the two compounds. Acid metarhodopsin bleaches above about 15°C.; rhodopsin is stable up to about 32°C.

TABLE IV

Thermal Bleaching of Rhodopsin and Acid Metarhodopsin

The Arrhenius energy (E_a), and the heat ($\Delta H\ddagger$), free energy ($\Delta F\ddagger$), and entropy ($\Delta S\ddagger$) of activation are derived from the velocity constant, k, and temperature, T, as described in the text.

I T	II k	III E_a	IV $\Delta H\ddagger$	V $\Delta F\ddagger$	VI $\Delta S\ddagger$
			Rhodopsin		
°K.	sec.$^{-1}$	cal./mole	cal./mole	cal./mole	cal./mole
308	0.0000404	71,800	71,200	24,300	+152.7
313	0.000290	71,800	71,200	23,600	+152.3
316.9	0.00119	71,800	71,200	23,000	+152.2
319.7	0.00320	71,800	71,200	22,600	+152.1
322	0.00621	71,800	71,200	22,300	+151.9
Averages.....		71,800	71,200	23,200	+152.2
			Acid metarhodopsin		
293	0.0000460	18,300	17,700	23,100	−18.5
298	0.0000768	27,000	26,400	23,200	+10.7
303	0.000213	35,700	35,100	23,000	+39.9
308	0.000564	35,700	35,100	22,800	+40.0
Averages.....		29,200	28,600	23,100	+17.8

Fig. 9 shows data on the thermal bleaching of both compounds, plotted in terms of the equation for a monomolecular reaction in the form,

$$k = \frac{2.303}{t_2 - t_1} \log \frac{(a - x)_1}{(a - x)_2}.$$

in which k is the velocity constant, and $(a - x)_1$ and $(a - x)_2$ are the concentrations of rhodopsin or acid metarhodopsin at times t_1 and t_2. Plotted in this way, the points fall on straight lines, indicating that in both cases thermal bleaching follows the course of a monomolecular reaction. The velocity constants at the various temperatures are given in the first two columns of Table IV.

In Fig. 9 the extinctions at λ_{max} (493 and 500 mμ) are used to represent the concentrations of rhodopsin and acid metarhodopsin. This procedure is legitimate only if

absorption by the products of thermal bleaching is negligible at these wavelengths. This was shown to be the case by cooling the solutions during the reaction to stop bleaching and immediately adding hydroxylamine, which reacts with the products of thermal bleaching to form retinene oxime. The oxime has no appreciable absorption above 450 mμ. If the products of thermal bleaching absorbed appreciably near 500 mμ, the extinction would fall upon addition of hydroxylamine. We find that addition of hydroxylamine has a negligible effect on the extinctions at λ_{max} of rhodopsin and acid metarhodopsin. These extinctions are therefore valid measures of concentration.

As can be seen from the velocity constants in Table IV (column II), the temperature dependence of the bleaching rate differs markedly for rhodopsin and acid metarhodopsin. For a comparable rise in temperature (about 15°), the bleaching rate of acid metarhodopsin increases by a factor of 12, while the bleaching of rhodopsin is accelerated about 150-fold. The energies of activation for the two processes must therefore be quite different. These have been calculated from the Arrhenius equation, in the form,

$$\log \frac{k_1}{k_2} = \frac{E_a}{2.303\ R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right),$$

in which E_a is the activation energy, R, the gas constant, and k_1 and k_2, the velocity constants for thermal bleaching at absolute temperatures T_1 and T_2.

As shown in Fig. 10, the data for the thermal bleaching of rhodopsin (log k *vs.* $1/T$) fall on a straight line of slope $\frac{E_a}{2.303\ R}$, corresponding to an activation energy of 71,800 cal. per mole. The data for acid metarhodopsin lie on a curve of increasing slope (dotted line). This is probably not experimental error, but appears to represent an increase in activation energy with temperature from about 18,000 to about 36,000 cal. per mole, due to an interplay of two reactions with very different activation energies, as discussed below. The average activation energy over the temperature range covered in the present experiment is 29,200 cal. per mole (solid line).

Thermal bleaching therfore has a *lower* temperature threshold and a *lower* activation energy with acid metarhodopsin than with rhodopsin. What does this mean? The interpretation is easier if one considers not only the Arrhenius activation energy, but the free energy and entropy changes accompanying the activation process. These are commonly referred to as the "free energy of activation" and "entropy of activation," designated by the symbols $\Delta F\ddagger$ and $\Delta S\ddagger$ (*cf.* Glasstone, Laidler, and Eyring, 1941, chapter 1).

These functions are derived from the velocity constant, on the assumption that the process of activation is a reversible reaction to which the laws of thermodynamics apply. The Arrhenius energy of activation (E_a) is related to the corresponding thermodynamic function, the heat of activation ($\Delta H\ddagger$), by the equation,

$$\Delta H\ddagger = E_a - RT.$$

The heat of activation is related to the free energy and entropy of activation by the familiar thermodynamic relationship,

$$\Delta F\ddagger = \Delta H\ddagger - T\Delta S\ddagger.$$

$\Delta F\ddagger$ and $\Delta S\ddagger$ are derived from the velocity constant, k, at temperature T by the equations (after Glasstone *et al.*, 1941, p. 14),

$$\Delta F\ddagger = 2.303\ RT \log \frac{k_B T}{kh},$$

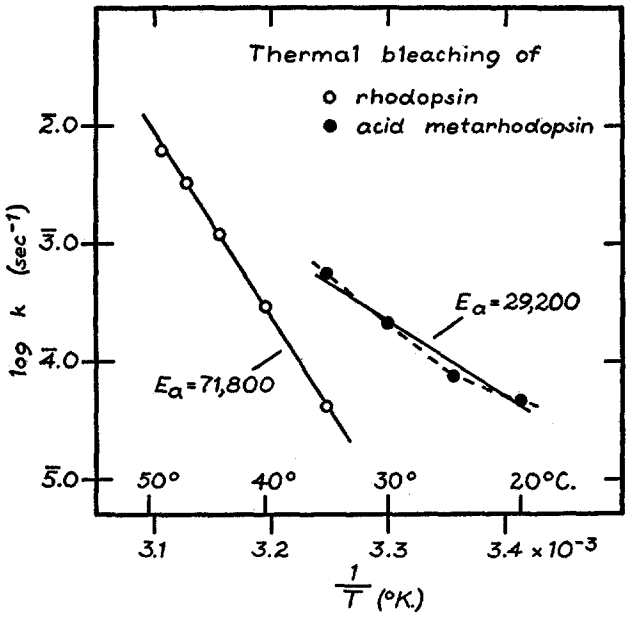


FIG. 10. Arrhenius activation energies (E_a) for the thermal bleaching of rhodopsin and acid metarhodopsin. E_a is calculated from the Arrhenius equation in the form, $\log \frac{k_1}{k_2} = \frac{E_a}{2.303R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right)$, in which R is the gas constant (1.986 cal. mole°), and k_1 and k_2 are the velocity constants at absolute temperatures T_1 and T_2. When log k is plotted as a function of T, E_a is 2.303 R times the slope of the line drawn through the points.

and

$$\Delta S\ddagger = 2.303\ R \log \frac{kh}{k_B T} + \frac{\Delta H\ddagger}{T},$$

in which R is the gas constant, k_B the Boltzmann constant (1.38×10^{-16} erg degree), and h Planck's constant (6.57×10^{-27} erg sec.). As can be seen from these equations, the rate of a chemical reaction is determined by the *free energy* of activation and not

by the heat of activation or the Arrhenius activation energy, which is the sum of the free energy and an *entropy* term.

The values of these functions for the thermal bleaching of rhodopsin and acid metarhodopsin are summarized in Table IV. $\Delta F\ddagger$ is very similar for the two reactions, which is the reason why both take place at comparable absolute temperatures. The differences in Arrhenius activation energy (E_a) are due primarily to differences in the *entropies of activation.* $\Delta S\ddagger$ for bleaching rhodopsin is about +152 entropy units. Although this represents a very large increase in entropy compared with most ordinary chemical reactions, it falls within the range of entropies of activation observed for the inactivation of enzymes and other protein denaturations (about 100 to 300 entropy units; Glasstone *et al.*, 1941, pp. 196, 442–447; Stearn, 1949, pp. 31–34). $\Delta S\ddagger$ for the thermal bleaching of acid metarhodopsin, however, starts small and negative at 20°C., then becomes positive and increases to 40 entropy units at 35°C. At higher temperatures, it may enter the range for protein denaturations, but this is a separate consideration (see below).

In spite of the fact that protein denaturations have very large activation energies (E_a or $\Delta H\ddagger$), they usually proceed at temperatures well below 100°C. This is because protein denaturations, in general, have large positive entropies of activation, so that $\Delta F\ddagger$, which is the difference between $\Delta H\ddagger$ and $T\Delta S\ddagger$, always has a value of about 22 to 26 kcal. per mole (Glasstone *et al.*, 1941, pp. 442–447; Stearn, 1949, p. 32).

$\Delta S\ddagger$ may be considered a measure of the configurational change which occurs during the activation process. Protein denaturations entail a randomization of the structure of the protein fabric, hence a large positive $\Delta S\ddagger$. Most simple chemical reactions entail smaller configurational changes. For these, $\Delta S\ddagger$ is consequently closer to zero, and positive or negative depending on whether the configuration of the activated complex is more or less probable than that of the reacting molecules (for discussion of $\Delta S\ddagger$ see Stearn, 1949, pp. 60–66).

The present experiments suggest that the thermal bleaching of rhodopsin is caused by the denaturation of the protein moiety, whereas the bleaching of acid metarhodopsin, particularly at 20°C., involves only minor changes in configuration, such as may be associated with the simple dissociation of the chromophore from opsin.

If it is true that thermally bleached metarhodopsin is dissociated but not denatured, it should be possible to resynthesize rhodopsin from neo-*b* retinene and thermally bleached acid metarhodopsin. As mentioned above, this is not the case. This failure to regenerate rhodopsin, however, does not necessarily invalidate the above conclusions. Opsin generally is denatured more readily than rhodopsin (Radding and Wald, 1955–56 *b*; unpublished experiments from this laboratory by Brown and by Dowling). One of us has recently studied the thermal denaturation of cattle rhodopsin and opsin (Hubbard, unpublished experiments). Cattle opsin is denatured above about 40°C., while rhodopsin is stable

up to about 60°C. Above 40°C. bleaching of cattle rhodopsin by light is followed immediately by the thermal denaturation of opsin and is therefore irreversible. If squid rhodopsin and opsin behave similarly, free opsin may denature above about 15°C. The thermal bleaching of acid metarhodopsin at 20°C. therefore, though due to the simple dissociation of retinene from opsin, may be rendered irreversible by the rapid denaturation of opsin. The rise with temperature of E_a and $\Delta S\ddagger$ suggests that at the higher temperatures, denaturation of the protein moiety becomes the primary cause of bleaching with acid metarhodopsin, just as with rhodopsin. The data on thermal bleaching of squid rhodopsin and acid metarhodopsin may therefore be summarized tentatively as follows:—

$$\text{rhodopsin} \xrightarrow{\text{above 35° C.}} \text{denatured opsin} + \text{retinene}$$

$$\text{Acid metarhodopsin} \xrightarrow{\text{20° C.}} \text{native opsin} + \text{retinene}$$

$$\text{native opsin} \downarrow \text{denatured opsin}$$

$$\text{Acid metarhodopsin} \xrightarrow{\text{above 30–35° C}} \text{denatured opsin} + \text{retinene}$$

Recapitulation.—Light interconverts squid rhodopsin and metarhodopsin. Both consist of retinene bound to opsin at the same site, but in rhodopsin the retinene is in the neo-*b* (11-*cis*) configuration, whereas in metarhodopsin it is all-*trans*. This difference in the *shapes* of the chromophores exerts a marked effect on the stabilities of the two compounds. Thus rhodopsin is much more resistant than metarhodopsin to chemical attack or heat.

3. Regeneration of Squid Rhodopsin

We showed above that the regeneration of squid rhodopsin from metarhodopsin in solution requires light and always yields a steady state mixture of rhodopsin and metarhodopsin. Squid retinas, however, contain rhodopsin uncontaminated with metarhodopsin. This is true to a first approximation, even when the animals have been kept in moderate light, or have been light-adapted with a photoflash lamp prior to decapitation in the dark. Yet isolated retinas or rod suspensions exposed to light form mixtures of rhodopsin and metarhodopsin. If we assume that light converts rhodopsin to metarhodopsin also *in vivo*, the squid retina must possess a mechanism for reconverting metarhodopsin to rhodopsin quantitatively and rapidly in the dark. We have been unable to demonstrate this reaction in isolated retinas, rods, or rhodopsin solutions.

What pathways are open for regenerating rhodopsin in the squid retina? It has been shown that the eyes of crustacea—the lobster and certain euphausiids—contain large amounts of vitamin A (Fisher, Kon, and Thompson,

1952, 1954, 1955), specifically in the form of the neo-*b* (11-*cis*) isomer (Wald and Burg, 1956–57; Wald and Brown, 1956–57). The squid eye contains very little vitamin A (Wald, 1941, 1942; Fisher, Kon, and Thompson, 1956)—only 5 to 10 per cent of its rhodopsin content on a molar basis—all of it in the form of the all-*trans* isomer. It contains also very little free retinene. About 90 per cent of its retinene is bound to protein in light or darkness (Wald, 1941, 1942).

The protein-bound retinene can be accounted for entirely as rhodopsin. We have determined protein-bound retinene and rhodopsin in aliquots of homogenized squid retinas. One experiment yielded 0.076 micromole rhodopsin, compared with 0.074 micromole protein-bound retinene; another yielded 0.023 micromole rhodopsin, as against 0.024 micromole protein-bound retinene. The squid eye therefore has essentially no stores of vitamin A or free retinene to tap for rhodopsin synthesis. The visual system must involve primarily interconversions of rhodopsin and metarhodopsin. The pH of the squid body fluids presumably is like that of sea water, which ranges between pH 7.5 and 8.4 (Sverdrup, Johnson, and Fleming, 1942, pp. 194–195, 209–210). The squid retina in the light therefore should contain both acid and alkaline metarhodopsin, either of which could be the physiological precursor of rhodopsin.

Clearly the squid must also possess some way of synthesizing rhodopsin from vitamin A or retinene, for these are its ultimate sources. The squid eye must therefore possess at least *two* mechanisms for rhodopsin synthesis in the dark: one, a rapid conversion of metarhodopsin to rhodopsin; the other, a possibly slow synthesis of chromoprotein (rhodopsin or metarhodopsin) from retinene and opsin. Neither of these has yet been demonstrated *in vitro*.

DISCUSSION

The prosthetic group of rhodopsin is neo-*b* retinene both in vertebrates and in the squid. But whereas light bleaches vertebrate rhodopsins to mixtures of all-*trans* retinene and opsin (Hubbard and Wald, 1952–53), it converts squid rhodopsin to the all-*trans* chromoprotein, squid metarhodopsin. Light therefore effects a stereoisomerization in both types of rhodopsin system.

We need not labor the point that a pigment, in order to be effective in vision, must be *changed* by visible light in some way that can be translated into a nervous excitation. Wald and coworkers have shown that light exposes new reactive groups on opsin which may be ultimately responsible for the initiation of the nerve impulse (Wald and Brown, 1951–52; Radding and Wald, 1955–56 *a*, *b*). We wish to suggest, however, that *the primary effect of light is the stereoisomerization of the chromophore to the all-trans configuration.* In vertebrates, this is followed by a series of now familiar dark reactions, whereby retinene is split from opsin (Wald, 1937–38; Lythgoe, 1938; Wald, Durell, and St. George, 1950). In the squid, under physiological conditions, there is no dark reaction. In other words, the primary difference between the squid and vertebrate rhodopsin systems involves the stability of metarhodopsin. In the squid it is rela-

tively stable, and the system appears ordinarily to involve no more than the reversible stereoisomerization of the chromophore in the reaction,

$$\text{Rhodopsin} \underset{\longleftarrow}{\overset{\text{light}}{\longrightarrow}} \text{metarhodopsin.}$$

In vertebrates the metarhodopsin is unstable and dissociates to retinene and opsin, followed by the reduction of retinene to vitamin A.

Collins (1953) and Morton and Pitt (1955) have shown that in cattle rhodopsin, retinene is attached to an amino group on opsin, forming a Schiff base, N-retinylidene–opsin,

$$\underset{\text{Retinene}}{C_{19}H_{27}\cdot\overset{H}{C}{=}O} + H_2N\cdot\text{opsin} \rightleftharpoons \underset{\text{Schiff base}}{C_{19}H_{27}\cdot\overset{H}{C}{=}N\cdot\text{opsin}} + H_2O.$$

The rhodopsin chromophore is thought to be derived from the conjugate acid of this Schiff base, $C_{19}H_{27}\cdot\overset{H}{\overset{+}{C}}{=}N\cdot\text{opsin}$ (Morton and Pitt, 1955; Hubbard, 1958 *b*). However, several lines of evidence show that the aldehyde group serves only to anchor retinene to opsin. Its entire hydrocarbon chain is involved in making the chromophore of rhodopsin or metarhodopsin (*cf.* Wald, 1956; Hubbard, 1958 *b*). Moreover, the geometric configuration of retinene determines its capacity to be fitted onto the surface of opsin, and hence its capacity to form a stable chromophore.

Fig. 11 illustrates schematically a possible difference between vertebrate and squid opsins which may account for their different chemical behavior. Let us assume that both types of opsin complement the shape of the neo-*b* chromophore from the Schiff base linkage at the right to the β-ionone ring at the left. With vertebrate opsins (Fig. 11, left) isomerization to the all-*trans* configuration pulls the chromophore away from the opsin surface, leaving it attached only by the Schiff base linkage. In neutral solution, such Schiff bases readily hydrolyze to the free aldehyde and the amine (Pitt *et al.*, 1955; Morton and Pitt, 1955), so releasing the chromophore as all-*trans* retinene. Squid opsin (Fig. 11, right), however may fit the geometry of both the neo-*b* and all-*trans* chromophores, and stabilize both against hydrolysis. Yet, as we have seen, even squid metarhodopsin is considerably less stable than rhodopsin.

These observations have implications for the excitation process. Electrical responses can be recorded from the receptor cells within milliseconds after the onset of light (*cf.* Hartline, 1954). Wald (1954) has therefore suggested that visual excitation probably depends upon the transformation of rhodopsin to lumi- or metarhodopsin, rather than upon the subsequent and much slower release of retinene from the chromophoric site.

It is known that light exposes new reactive groups on opsin which may play a significant part in nervous excitation (Wald and Brown, 1951–52; Radding

and Wald, 1955–56 *a*, *b*; Wald, 1956). The irradiation of cattle rhodopsin *immediately* exposes one titratable group with pK 6.6 (Radding and Wald, 1955–56 *a*), while irradiation of squid rhodopsin exposes one titratable group with pK 7.7. These pK's are at or just below the pH's of the respective body fluids. The exposure of these groups may be ultimately responsible for triggering the propagated impulse.

Can we identify the ionizable group with pK 7.7 in squid metarhodopsin? Ball *et al.* (1949) have shown that the Schiff bases of retinene are pH indicators

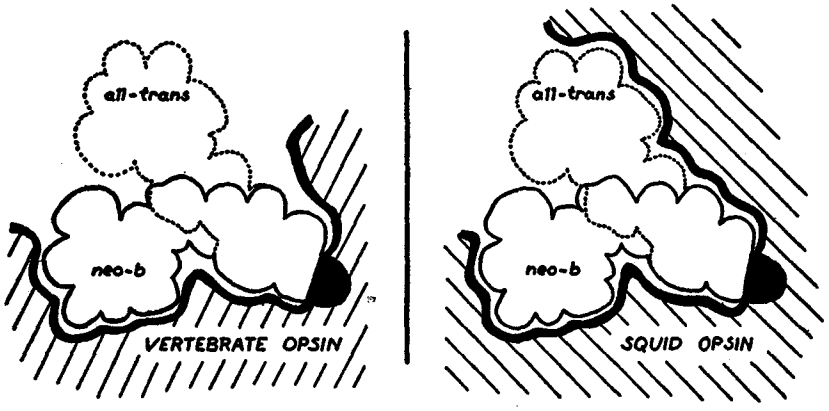


FIG. 11. Steric fit between retinene chromophores and vertebrate and squid opsins. Hypothetical configurations of the chromophoric sites on vertebrate and squid opsin. Both opsins complement the shape of the neo-*b* chromophore all the way from the Schiff base linkage at the right to the β-ionone ring at the left. But while squid opsin (right) also complements the shape of the all-*trans* chromophore, vertebrate opsin (left) does not.

since addition of a hydrogen ion to the —C=N— linkage shifts the absorption spectrum toward longer wavelengths. The pH indicator properties of squid metarhodopsin are apparently associated with the titration of the Schiff base linkage (see p. 507):

$$\underset{\text{Alkaline metarhodopsin}}{C_{19}H_{27}\cdot\overset{H}{C}{=}N\cdot\text{opsin}} + H^+ \rightleftharpoons \underset{\text{Acid metarhodopsin}}{C_{19}H_{27}\cdot\overset{H}{\overset{+}{C}}{=}N\cdot\text{opsin}.}$$

The study of simple Schiff base models of the rhodopsin chromophore has been haunted by the notion that a proper analog should bleach in the light (*cf.* Pitt *et al.*, 1955). We have suggested that the photosensitivity of rhodopsin is due to the fact that light isomerizes the neo-*b* chromophore to the all-*trans* configuration. Bleaching *per se* is not a necessary concomitant of this photoisomerization, but follows only if the isomerized chromophore does not fit the

opsin surface. When such fitting of the hydrocarbon chain of retinene is not involved—as in structurally simple analogs—there is no reason to expect bleaching.

In conclusion, we note that for the process of vision, the eye has selected a reaction in which the direct effect of light is to change the *shape* of the prosthetic group of a chromoprotein, so changing its relationship with the protein surface. We have come to expect proteins to impose stringent steric requirements in their interactions with other molecules. The stereospecificity of opsin provides a mechanism for translating the absorption of quanta of light instantaneously into a chemical reaction, which may then be communicated as the physiological event of visual excitation.

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